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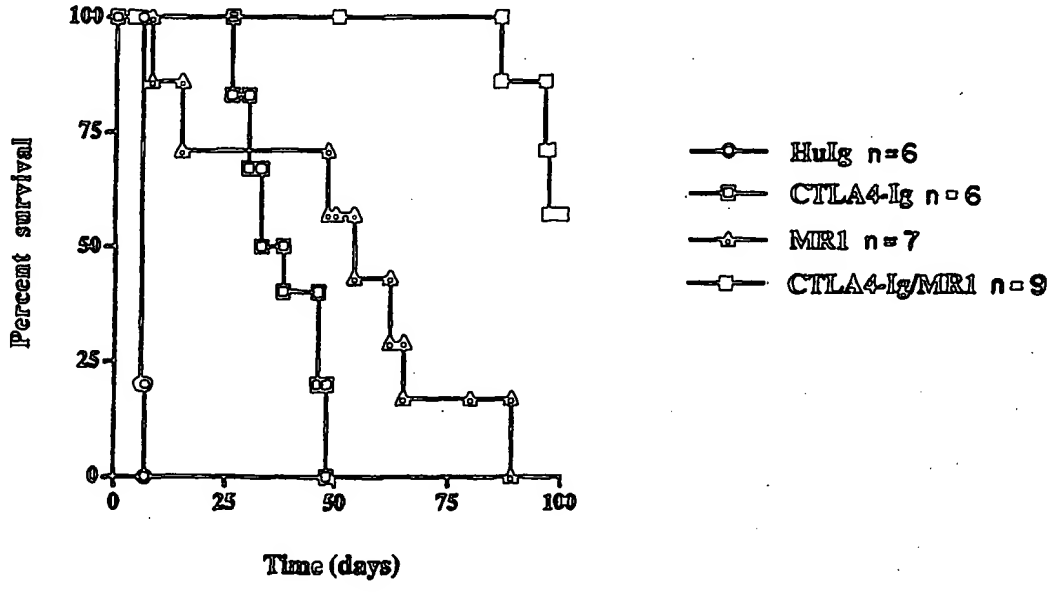


(57) Abstract

The present invention provides a method for inhibiting an immune response and a method for inhibiting rejection of transplanted tissues. This method comprises preventing an endogenous molecule on a cell selected from the group consisting of gp39 and CD40 antigens from binding its endogenous ligand and preventing an endogenous molecule on a cell selected from the group consisting of CTLA4, CD28, and B7 antigens from binding its endogenous ligand. The prevention of such molecules from binding their ligand thereby blocks two independent signal pathways and inhibits the immune response resulting in transplanted tissue rejection.

METHODS FOR INHIBITING AN IMMUNE RESPONSE BY BLOCKING THE GP39/CD40 AND CTLA4/CD28/B7 PATHWAYS AND COMPOSITIONS FOR USE THEREWITH

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION

CD28 is expressed on most T lineage cells and plasma cells (June, C.H. et al., Immunol. Today 11, 211-16 (1990); Damle et al., Proc. Natl. Acad. Sci. 78:5096-6001 (1981)). The ligand for CD28 is B7, which is expressed on activated B cells (Linsley, P.S. et al., Proc. Natl. Acad. Sci. USA 87, 5031-35 (1990); Linsley, P.S. et al., J. Exp. Med. 173, 721-730 (1991).

CD40 is a member of the tumor necrosis factor receptor (TNFR) family of type I membrane-bound signaling receptors. Though originally identified as a B cell antigen, CD40 is expressed by all antigen presenting cells (APC) including dendritic cells, monocytes, and B cells.

The ligand for CD40 is gp39, which binds to CD40 and thus can activate B cells. Gp39 is also known as CD40L, TRAP and T-BAM. Gp39 is a type II cell surface protein with significant homology to TNF and is transiently expressed by activated T cells. In addition to T cells, gp39 is expressed by basophils, mast cells, and eosinophils.

The CD28 and CD40 pathways play essential roles in the initiation and amplification of T-dependent immune responses (Bluestone, J.A. Immunity 2, 555-9 (1995); Banchereau J., et al. Own. Rev. Immunol. 12, 881-922 (1994); Durie, F.H., et al. Science 261, 1328-30 (1993); Foy, T.M., et al. J Exp Med 178, 1567-75 (1993); Van den Eertwegh, A.J.M., et al. J Exp Med 178, 1555-65 (1993)).

CD28/B7 interactions provide critical "second signals" necessary for optimal T cell activation, and IL-2 production (Jenkins, M.K., et al. J. Immunol. 147, 2461-6 (1991); Schwartz, R.H. Cell 71, 1065-8 (1992); Boussiotis, V.A., et al. J. Exp. Med. 178,

Presently, there exists a need to provide ways to effect long-term tolerance of transplanted tissues by the host, thereby increasing the survival rate of transplantation. To do so, it is necessary to ensure sufficient immunologic unresponsiveness in the transplant recipient.

We have found that the inhibition of T-dependent immune responses resulting from blockade of either CD28 or CD40 signals is potent, but incomplete. The data herein demonstrate that simultaneous blockade of these pathways unexpectedly inhibits acute and chronic rejection of transplanted tissue in vivo. Independent blockade of these pathways using a soluble CTLA4 molecule or antibodies which recognize and bind gp39 failed to even minimally prolong survival of primary skin transplanted tissue.

The invention herein involves the discovery that simultaneous blockade of CD28 and CD40 signals promoted long-term survival of fully allogeneic as well as xenogeneic skin grafts. Prolongation of skin allograft survival was eliminated by cyclosporine A (CyA), suggesting that it is an active process requiring intact signaling via the TcR/CD3 complex and/or other CyA sensitive pathways. Moreover, CTLA4Ig/MR1 promoted long-term acceptance of primarily vascularized cardiac graft tissue, and inhibited the development of chronic vascular rejection.

The effect demonstrated in the two transplantation models herein indicates that CD28 and CD40 provide interrelated, yet independent signaling pathways required for the generation of effective T cell responses. This discovery provides methods which are new and more effective strategies to manipulate immune responses including suppressing graft rejection.

SUMMARY OF THE INVENTION

The present invention provides a method for inhibiting rejection of a transplanted tissue. This method comprises preventing an endogenous molecule (e.g., antigen) on a cell selected from the group consisting of gp39 and CD40 from binding its endogenous ligand and preventing an endogenous molecule on a cell selected from the group consisting of CTLA4, CD28, and B7 from binding its endogenous ligand. The prevention of such molecules from binding their ligands thereby blocks two independent signal pathways and inhibits the immune response responsible for transplanted tissue rejection.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a bar graph showing that simultaneous blockade of CD28 and CD40 signals ablate popliteal lymph node alloimmune responses in vivo.

Figure 2A is a line graph that shows CTLA4Ig/MR1 treatment prolongs cardiac allograft survival in comparison with CTLA4Ig or MR1 alone.

Figure 2B is a photograph of a histologic section showing CTLA4Ig-treated cardiac allograft at day 62 having extensive lymphocytic infiltration, interstitial fibrosis, and severe coronary arterial intimal thickening and fibrosis consistent with chronic rejection (left panel 100X magnification; right panel 400X magnification).

Figure 2C is a photograph of a histologic section showing a MR1-treated cardiac allograft at day 62 having less lymphocytic infiltration and interstitial fibrosis, but severe coronary vasculopathy characteristic of chronic rejection (left panel 100X magnification; right panel 400X magnification).

Figure 2D is a photograph of a histologic section showing CTLA4Ig/MR1-treated cardiac allografts at day 58, free from lymphocytic infiltration, fibrosis, coronary arterial intimal lesions (left panel 100X magnification; right panel 400X magnification).

Figure 2E is a photograph of a histologic section showing normal untransplanted BALB/c hearts (left panel 100X magnification; right panel 400X magnification).

Figure 3A is a photograph of ethidium bromide stained gel strips showing intragraft expression of immune mediator transcripts using RT-PCR in untreated, MR1 treated, CTLA4Ig treated, and MR1/CTLA4Ig treated cardiac allografts.

Figure 3B is a series of bar graphs showing the mean PCR product band intensities ± standard deviation.

Figure 4A is a line graph showing data of mice treated with MR1 alone, CTLA4Ig alone, and a combination of MR1 and CTLA4Ig.

Figure 6C is a bar graph showing that simultaneous blockade of the CD40 and CD28 pathways markedly inhibits cytokine production of IL-2. Human IgG (stippled), CTLA4-Ig (gray), MR1 (white), CTLA4-Ig/MR1 (black), normal unimmunized node (hatched).

Figure 6D is a bar graph showing that simultaneous blockade of the CD40 and CD28 pathways markedly inhibits cytokine production of INFg. Human IgG (stippled), CTLA4-Ig (gray), MR1 (white), CTLA4-Ig/MR1 (black), normal unimmunized node (hatched).

Figure 7A is a line graph showing that C3H recipients treated with CTLA4-Ig (500 μg) on days 0, 2, 4 and 6 combined with MR1 (500 μg) on days 0, 2, 4 and 6 had prolonged survival of Sprague-Dawley rat cardiac allografts.

Figure 7B is a photograph of an untreated cardiac xenograft at day 6 showing widespread tissue destruction (400X).

Figure 7C is a photograph of a CTLA4-Ig treated cardiac xenograft at day 20 showing lymphocytic infiltration, myocyte destruction, and coronary vasculopathy (400X).

Figure 7D is a photograph of a MR1 treated cardiac xenograft at day 20 showing lymphocytic infiltration, myocyte destruction, and coronary vasculopathy (400X).

Figure 7E is a photograph of a normal untransplanted Sprague-Dawley rat heart (400X).

Figure 7F is a photograph of a CTLA4-Ig/MR1 treated cardiac xenograft at day 20, essentially free from lymphocytic infiltration and fibrosis (400X).

Figure 7G is a photograph of a CTLA4-Ig/MR1 treated cardiac xenograft at day 122, demonstrating excellent preservation of both myocytes and vascular structures (400X).

Figure 8A is a series of line graphs showing prolongation of Sprague-Dawley rat skin xenograft survival in C3H mice treated with MR1 and CTLA4-Ig administered together in the perioperative period as compared with xenograft recipients treated with either MR1 alone or CTLA4-Ig alone and untreated controls.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

All scientific and technical terms used in this application have meanings commonly used in the art unless otherwise specified. As used in this application, the following words or phrases have the meanings specified.

As used herein "monoclonal antibodies directed against gp39" or "anti-gp39" includes MR1. Anti-gp39 is also known in the literature as an antiCD40 ligand. Examples of MR1 include, but are not limited to monoclonal antibodies directed against gp39 from mouse; antibodies directed against gp39 from other species such as monkey, sheep, human are included. Additionally, "monoclonal antibodies directed against gp39" or "anti-gp39" includes any antibody molecule, fragment thereof, or recombinant binding protein that recognizes and binds gp39.

As used herein, "administering" means oral administration, administration as a suppository, topical contact, intravenous, intraperitoneal, intramuscular or subcutaneous administration, or the implantation of a slow-release device such as a miniosmotic pump, to the subject.

As used herein, "pharmaceutically acceptable carrier" includes any material which when combined with the antibody retains the antibody's immunogenicity and is non-reactive with the subject's immune systems. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets including coated tablets and capsules.

Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

As used herein, "transplanted tissue" includes autografts, isografts, allografts, and xenografts. Examples of transplanted tissue include, but are not limited to, solid

In another example, endogenous CD40 antigen is prevented from binding its endogenous ligand. This example comprises the step of contacting a CD40-positive cell with a soluble ligand which recognizes and binds the CD40 antigen. Suitable ligands include antibodies directed against CD40 or soluble gp39 (sgp39).

This example comprises the additional step of preventing the endogenous CTLA4 antigen from binding its endogenous ligand. This step comprises contacting a B7-positive cell with a soluble ligand which recognizes and binds the B7 antigen. Examples of this soluble ligand include CTLA4Ig, soluble CD28 molecules, and antibodies directed against B7.

The binding of the CD40-positive cell to its soluble ligand blocks the reaction of endogenous CD40 antigen with endogenous gp39. The binding of the B7-positive cell to its soluble ligand blocks the reaction of the B7 antigen with endogenous CTLA4. The combined blockage inhibits the immune response.

In yet another example, endogenous gp39 antigen is prevented from binding its endogenous ligand as described above. The example comprises the additional step of preventing the endogenous CD28 antigen from binding its endogenous ligand. This step comprises contacting a B7-positive cell with a soluble ligand which recognizes and binds the B7 antigen. Examples include CTLA4Ig, soluble CD28 molecules, and antibodies directed against B7 such as BB-1.

The binding of the gp39-positive cell to its soluble ligand blocks the reaction of gp39 antigen with endogenous CD40. The binding of the B7-positive cell to its soluble ligand blocks the reaction of the B7 antigen with endogenous CD28. This combined blockage inhibits the immune response.

In another example, endogenous CD40 antigen is prevented from binding its endogenous ligand as described above. The example provides the additional step of preventing the endogenous B7 antigen from binding its endogenous ligand. This comprises contacting a CD28-positive cell with a soluble ligand which recognizes and binds the CD28 antigen. Examples of such soluble ligands include soluble B7 molecules and antibodies directed against CD28.

The binding of the CD40-positive cell to the soluble ligand blocks the reaction of CD40 antigen with endogenous gp39. The binding of the CD28-positive cell to the

Additionally, the present invention provides another embodiment for a method for inhibiting an immune response resulting in graft rejection. This embodiment comprises contacting a B7-positive cell with a first soluble ligand which recognizes and binds the B7 antigen, and contacting a gp39-positive cell with a second soluble ligand which recognizes and binds the gp39 antigen.

The binding of the B7-positive cell to the first soluble ligand blocks the reaction of the B7 antigen with endogenous CTLA4 or CD28. Further, the binding of the gp39 antigen to the second soluble ligand blocks the reaction of gp39 antigen with endogenous CD40. The combination of this blockage inhibits the immune response.

Additionally, the invention provides a method for inhibiting an immune response mediated by the CTLA4/CD28/B7 and gp39/CD40 pathways in a subject. In accordance with the practice of the invention, the subject may be an animal subject such as a human, a dog, a cat, a sheep, a horse, a mouse, a pig, or a cow.

The method comprises administering to the subject a first soluble ligand which recognizes and binds the B7 antigen (e.g. soluble CTLA4 or CD28 molecules) and a second soluble ligand which recognizes and binds the gp39 antigen (e.g., monoclonal antibodies directed against gp39 (MR1) or soluble CD40 molecules). The binding of the first and second ligands to their receptor inhibits the immune response mediated by CTLA4-, CD28-, and gp39- cell interactions with B7- and CD40-positive cells.

Also, the invention provides a method for inhibiting transplant rejection in a subject. This method comprises administering to the subject an effective amount of a combination of a first soluble ligand which recognizes and binds the B7 antigen on B7-positive cells and a second soluble ligand which recognizes and binds the gp39 antigen on gp39-positive cells. The binding of B7-positive cells with the first soluble ligand and gp39-positive cells with the second soluble ligand disrupts endogenous CTLA4-, CD28-, and gp39- cell interactions with B7-positive cells and gp39-positive cells so that transplant rejection is inhibited.

In accordance with the practice of the invention, the first soluble ligand may be a recombinant binding molecule having at least a portion of the extracellular domain of CTLA4. In accordance with the practice of the invention, the extracellular portion of

In one example, the second binding domain is a ligand which recognizes and binds CTLA4. Examples include B7 and monoclonal antibodies directed against CTLA4. In another example, the second binding domain is a ligand which recognizes and binds the CD28 antigen. Examples include B7 and monoclonal antibodies directed against CD28. In another example, the second binding domain is a ligand which recognizes and binds the B7 antigen. Examples include CTLA4, CD28 and monoclonal antibodies directed against B7.

Soluble ligands may be administered during transplant, before transplant, or after transplant. Soluble ligands may be administered by oral means, transdermal means, intravenous means, intramuscular means, intraperitoneal, or by subcutaneous administration.

The most effective mode of administration and dosage regimen for the molecules of the present invention depends upon the location of the tissue or disease being treated, the severity and course of the medical disorder, the subject's health and response to treatment and the judgment of the treating physician. Accordingly, the dosages of the molecules should be titrated to the individual subject.

By way of example, the interrelationship of dosages for animals of various sizes and species and humans based on mg/m2 of surface area is described by Freireich, E.J., et al. Cancer Chemother., Rep. 50 (4): 219-244 (1966). Adjustments in the dosage regimen may be made to optimize suppression of the immune response resulting in graft rejection, e.g., doses may be divided and administered on a daily basis or the dose reduced proportionally depending upon the situation (e.g., several divided doses may be administered daily or proportionally reduced depending on the specific therapeutic situation).

It would be clear that the dose of the composition of the invention required to achieve an appropriate clinical outcome may be further reduced with schedule optimization.

The present invention also provides pharmaceutical compositions useful in inhibiting graft rejection or in inhibiting an immune response. In one embodiment, these compositions comprise an effective amount of a combination of (a) soluble ligands which recognize and bind any one of CTLA4, CD28, and B7 antigens, together with (b) soluble ligands which recognize and bind any one of gp39 and CD40 antigens and an acceptable carrier. In another embodiment, these compositions comprise an

DISCUSSION

Five days after subcutaneous immunization with allogeneic splenocytes, the draining popliteal lymph nodes on the side of antigen challenge underwent a >5 fold increase in weight relative to the contralateral node in untreated control mice. Treatment with either CTLA4Ig or MR1 resulted in a 50-60% inhibition of the response, whereas concomitant administration of CTLA4Ig and MR1 ablated lymph node expansion in response to antigen challenge. The results represent the mean ± standard deviation for 3 individual mice in each group. Similar results were obtained in three independent experiments.

Control mice demonstrated a 4-6 fold increase in the weight of the node draining the immunized foot relative to the node draining the contralateral foot injected with sterile saline (Figure 1). This increase in weight was accompanied by a dramatic expansion of the lymphocyte-rich paracortical (T cell) and cortical (B cell) regions. When administered alone, CTLA4Ig and MR1 each produced partial inhibition of this response (57% and 56% inhibition, respectively). The combination of CTLA4Ig/MR1 ablated lymph node expansion (98% inhibition, Figure 1) and prevented expansion of the paracortical and lymphoid follicles.

EXAMPLE 2

This example shows prolongation of cardiac allograft survival and inhibition of vasculopathy associated with chronic rejection.

METHOD

Male C3H/HeJ mice were transplanted with primarily vascularized BALB/c heart allografts at 8-12 weeks of age using microsurgical techniques (Corry, R.J., Winn, H.J. & Russell, P.S. Transplantation 16, 343-350 (1973)).

Rejection was defined by the loss of palpable cardiac contractions with confirmation at laparotomy by direct visualization. At specified times after transplant, the transplanted hearts were excised, formalin fixed and embedded in paraffin. Tissue sections (5 mm) were stained with Masson's Trichrome or hematoxylin-eosin. Each histologic specimen was reviewed by a cardiac transplant pathologist (KJW) blinded to the treatment modality.

fibrosis consistent with chronic rejection (Figure 2B). While the MR1-treated allograft demonstrated less lymphocytic infiltration and interstitial fibrosis, these grafts also had severe coronary vasculopathy characteristic of chronic rejection (Figure 2C).

In marked contrast, the allograft from CTLA4Ig/MR1 treated recipients were remarkably free from lymphocytic infiltration, fibrosis, and most significantly, coronary arterial intimal lesions (Figure 2D). In fact, the parenchyma and blood vessels of these grafts were virtually indistinguishable from those found in normal BALB/c hearts (Figure 2E).

EXAMPLE 3

This example shows blockade of T cell cytokine and costimulatory molecule transcript expression.

METHOD

At 8 days after transplantation, the cardiac grafts were removed and total RNA was prepared from tissues using TRIzol Reagent (GIBCO BRL, Gaithersburg, MD). cDNA was synthesized using 5 mg of total RNA template with a Superscript Preamplification System (GIBCO BRL, Gaithersburg, MD) in a final volume of 20 ml. PCR reactions were carried out. PCR products were visualized on ethidium bromide stained 1% agarose (BIO-RAD, Hercules, CA), 2% NuSieve GTG agarose (FMC BioProducts, Rockland, ME) gels. Gel images were stored using a UVP Gel Documentation System 5000. Band intensity was quantified using Gelreader analysis software (National Center for Supercomputing Applications, Urbana, IL).

In Figure 3A, intragraft expression of immune mediator transcripts was assessed using RT-PCR in untreated, MR1-treated, CTLA4Ig treated, and MR1/CTLA4Ig treated cardiac allografts.

Three allografts from each treatment group and the control group were analyzed at 8 days post-transplant. Normal heart tissue (N) and a syngeneic heart graft (S) at 8 days after transplantation were included for comparison.

METHOD

Segments of either full thickness tail or ear skin of approximately 1 cm square were grafted on to the posterior-lateral thoracic wall of recipient mice and secured in place with a circumferential Bandaid®. The grafts were then followed by daily visual inspection. Rejection was defined as the complete loss of visible epidermal graft tissue. Treatment protocols for MR1 and CTLA4Ig were as detailed for heart transplant recipients in Figure 1. CyA (Sandoz, East Hanover, NJ) at a concentration of 50 mg/ml was administered at a rate of 0.5 ml/hr (~20 mg/kg/day) for 14 days via an osmotic pump (Alzet Model No. 2002, Alza, Palto Alto, CA) which was implanted subcutaneously in the dorsal region of the recipient at the time of skin grafting and removed at 21 days after transplant (Pereira, G.M., Miller, J.F. & Shevach, E.M. J Immunol 144, 2109-2116 (1990)). After sacrifice, the skin graft was excised, formalin fixed and embedded in paraffin. Tissue sections (5 mm) were stained with hematoxylin-eosin.

In Figure 4A, C3H/HeJ recipients treated with either MR1 alone (MST = 13 days, n = 5) or CTLA4Ig alone (MST = 12 days, n = 7) rejected fully MHC-disparate BALB/c skin grafts at the same rate as an untreated control group (MST = 13 days, n = 5). In contrast, when MR1 and CTLA4Ig were administered together in the perioperative period, the allografts enjoyed markedly prolonged survival (MST >50, n = 15).

In Figure 4B, mice treated with CyA alone (MST = 30 days, n = 4), CyA plus CTLA4Ig (MST = 30 days, n = 5), or CyA and MR1 (MST = 32 days, n = 4) all displayed similar modestly prolonged skin graft survival. Surprisingly, the salutary effect of CTLA4Ig/MR1 on skin graft survival was abolished by concomitant cyclosporine administration (MST = 34 days, n = 4).

In Figure 4C, C3H recipients of BALB/c skin grafts were not treated (MST 10d, n=3), or treated with MR1 (MST 13d, n=3), YTS191.1 (MST 14d, n=6), YTS191.1 and MR1 (MST 16d, n=6), YTS191.1 and CTLA4Ig (MST 19d, n=5), or CTLA4Ig and MR1 (MST > 50d, n=22). Thus far, >53 mice have been treated with CTLA4Ig/MR1. Of these, 2 died on days 13 and 21. All others have remained healthy throughout the experiments without signs of weight loss, infection, or malignancy.

follicles and adnexal structures (Figure 4F). Surprisingly, the salutary effect of CTLA4Ig/MR1 on skin graft survival was abolished by concomitant cyclosporine administration (Figure 4B).

The remarkable potency of this effect was most clearly evident in the primary skin allograft model. Neither CTLA4Ig or MR1 alone or with CyA significantly prolonged skin allograft survival. Only the combination of CTLA4Ig and MR1 produced >50 day survival of fully-MHC mismatched skin allografts. Similar prolongation in this stringent test of inhibition of the alloimmune response has previously only been observed using vigorous cytoablative and/or hematopoietic chimerism-based strategies (Mayumi, H. & Good, R.A. J Exp Med 169, 213-238 (1990); Ildstad, S.T. & Sachs, D.H., Nature 307, 168-170 (1984); Ilstad, S.T., et al. J Exp Med 162, 231-44 (1985); Cobbold, S.P., Martin, G., et al. Nature 323, 164-166 (1986); Qin, S., et al. Science 259, 974-977 (1993)).

EXAMPLE 5

To explore the effect of blockade of the CD28 and CD40 pathways on T cell proliferation, we studied the primary allogeneic mixed leukocyte reaction using T cells from both Iek-restricted pigeon cytochrome c-reactive (pcc-TCRTg) and Ld-alloreactive (2C) T cell receptor transgenic mice (REF HED and LOH). CTLA4Ig, a fusion protein which binds to the ligands for CD28 and its homologue CTLA4, effectively inhibited proliferation of all three T cell populations (Figure 5A).

In contrast, blockade of the CD40 pathway with the hamster anti-gp39 mAb, MR1, modestly (~50%) inhibited the proliferation of C3H/HeJ T cells responding to BALB/c dendritic cells, dramatically inhibited (³85%) pcc-TCRTg T cells to reacting to cytochrome c, but had negligible effects on the proliferation of 2C T cells responding to Ld-bearing BALB/c dendritic cells (Figure 5A).

Furthermore, simultaneous blockade with these agents cooperatively inhibited T cell proliferation in allogeneic mixed leukocyte reactions and pcc-TCRTg T cells, whereas MR-1 had no effect or slightly augmented the proliferation of 2C T cells when combined with CTLA4Ig (Figure 5A). These results indicate that not all T cells are dependent on CD40 signals for clonal expansion and may explain the inability of CD40 blockade to completely inhibit allograft rejection.

analysis with ELISA. Each point on all of the graphs represents the mean ± standard deviation of 5 mice per group. The experiment was repeated with similar results.

CYTOKINE ELISA. Sandwich ELISA was performed using paired antibodies {anti-IL-2, anti-IFN-gamma, anti-IL-2 biotin, anti-IL-4 biotin, anti-IFN-gamma biotin (Pharmingen, San Diego, CA), anti IL4 (kind gift from Peter Jensen)} and streptavidin-HRP (Pierce, Rockford, IL). Colorimetric detection was assayed using TMB substrate (Pierce). Data were collected using a SpectraMax plate reader and plotted as absorbance (370 nm) +/- sem. Standard curves for each cytokine were generated using recombinant cytokine (rIL2, Boehringer Mannheim, Indianapolis, IN; rIL4, R&D Systems, Minneapolis, MN; and rIFN-gamma, Biosource International, Camarillo, CA).

MICE. Male C3H/HeJ (H-2k) and DBA/2 (H-2d) mice and Sprague-Dawley rats were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 8-12 weeks of age.

CARDIAC TRANSPLANTATION. C3H/HeJ or DBA mice were transplanted with primarily vascularized Sprague-Dawley rat heart xenografts and monitored for rejection as described in Larsen C.P., Alexander D.Z., Hollenbaugh D., et al., Transplantation, 61(1):4-9 (1996) and Corry R.J., Winn H.J., Russell P.S., Transplantation, 16(4):343-350 (1973). Recipients were treated with 500 mg CTLA4-Ig combined with 500 mg MR1 on days 0, 2, 4 and 6. Control groups included recipients treated with CTLA4-Ig alone, MR1 alone or Human Ig. Paraffin embedded tissue sections (5 μm) were stained with Masson's Trichrome or hematoxylin-eosin. Histologic specimens were reviewed by a cardiac transplant pathologist (KJW) blinded to the treatment modality.

SKIN TRANSPLANTATION. Full thickness skin grafts (~ 1 cm2) from Sprague-Dawley rats were transplanted on the dorsal thorax of C3H recipient mice and survival followed by daily visual inspection. Rejection was defined as the complete loss of visible epidermal graft tissue. Control groups included recipients treated with: CTLA4-Ig alone; MR1 alone; and Human Ig. Two additional mice in each experimental group were sacrificed at 20 days post transplant for histologic analysis.

The results of the lymph node assays suggested that simultaneous blockade of the B7/CD28 and CD40/gp39 pathways would inhibit xenograft rejection. To explore this hypothesis we studied a vascularized cardiac xenograft model using Sprague-Dawley rats as donors and C3H/HeJ mice as recipients. Treatment with either CTLA4-Ig (MST=33 days) or MR1 (MST=51 days) alone prolonged xenograft survival when compared to untreated controls (MST=6 days) (Fig. 7A). However, CTLA4-Ig/MR1 in combination markedly prolonged survival (MST=104.5 days).

When examined histologically at 20 days post-transplant, xenografts treated with either CTLA4-Ig alone (Fig. 7C) or MR1 alone (Fig. 7D) showed heavy lymphocytic infiltration with evidence of myocyte destruction and vasculopathy consistent with moderate to severe cellular rejection. In sharp contrast, the xenografts from CTLA4-Ig/MR1 treated recipients were essentially free from lymphocytic infiltration, interstitial fibrosis, and coronary arterial intimal lesions (Fig. 7F). CTLA4-Ig/MR1-treated cardiac xenografts demonstrated excellent preservation of both myocytes and vascular structures at day 122 (Fig. 7G). Untreated xenografts showed widespread tissue destruction at day 6 (Fig. 7B). A normal untransplanted Sprague-Dawley rat heart is shown in Fig. 7E.

As a more stringent test of the ability of CD40/CD28 blockade to inhibit xenogeneic immune responses, we studied the effects of short term CD28 and /or CD40 blockade, on primary skin xenograft survival in mice.

C3H recipients treated with either MR1 (MST=11.5 days n=4) or CTLA4-Ig (MST=12 days n=4) alone rejected full thickness skin grafts from Sprague-Dawley rats at the same rate as untreated controls (untreated controls MST=11.5 days n=8) (Fig. 8A). In contrast, the skin xenografts on recipients treated with simultaneous MR1 and CTLA4-Ig in the perioperative period, demonstrated markedly prolonged survival (MST=53 days n=25) (Fig. 8A). A total of 25 mice received xenografts and treatment with CTLA4-Ig/MR1. With the exception of one mouse that died on day 4, all others have remained healthy throughout the experiments without signs of weight loss, infection, or malignancy.

Chronic treatment (beginning after the standard 4 dose regimen) with either the CTLA4-Ig/MR1 combination (500 μg of both agents weekly until day 100 or rejection, whichever came first) or MR1 (500 μg of MR1 weekly until day 100 or rejection) resulted in no significant change in xenograft survival (Fig. 8B).

DISCUSSION

Combined blockade of the CD28 and CD40 pathways markedly inhibits the immune response to xenoantigen. The potency of this combination therapy was particularly demonstrated in the primary skin xenograft model. Neither agent alone prolonged skin xenograft survival, while, in contrast, the simultaneous combination of CTLA4-Ig and MR1 cooperatively inhibited xenograft rejection. The uniqueness of the findings resides in the stringency of the skin graft model, as CTLA4-Ig alone has previously been shown to prolong the survival of xenogeneic pancreatic islets in a mouse model, Lenschow D., Zeng Y., Thistlethwaite J., et al., Science, 257:789-792 (1992). Long-term survival of xenogenic skin grafts have previously only been observed using vigorous cytoablative and/or hematopoeitic chimerism–based strategies, Ildstad S.T., Sachs D.H., Nature, 307:168-170 (1984), Zhao Y., Swenson K., Sergio J., Arn J.S., Sachs D.H., Sykes M., Nat. Med., 2(11):1211-1216 (1996), Mayumi H., Good R.A., J. Exp. Med., 169(1):213-238 (1990), Cobbold S.P., Martin G., Zin S., Waldman H., Nature, 323:164-166 (1986), Qin S., Cobbold S., Benjamin R., Waldmann H., J. Exp. Med., 169:779-794 (1989).

The observation that simultaneous CD28/CD40 blockade can dramatically prolong xenograft survival suggests that both the antibody and cell mediated mechanisms for destruction of the xenograft may be effectively inhibited by this strategy. While the etiology of acute vacular xenograft rejection remains to be completely defined, there is evidence that it is caused, at least in part, by the development of xenoreactive antibodies (Cotterell A.H., Collins B.H., Parker W., Harland R.C., Platt J.L., Transplantation, 60(8):861-868 (1995)). The rapid destruction of untreated control cardiac xenografts in our model, in the absence of a cellular infiltrate, suggests a role for antibody mediated rejection. This observation and those of others (Aksentijevich I., Sachs D.H., Sykes M., Transplantation, 53(5):1108-14 (1992)), combined with the documented dramatic inhibition of the evoked xenoantibody response after blockade of the CD28 and CD40 pathways (Figures 9A and 9B), supports the hypothesis that xenoresponses may be sufficiently controlled by inhibition of these pathways to permit the development of non-cytoablative strategies for xenotransplantation in discordant species combinations.

While combined blockade of the CD28 and CD40 pathways markedly inhibited the xenograft rejection response, this blockade did not achieve uniform indefinite cardiac

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page ______, line ______

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
May 31, 1991	68629

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on
Authorized officer: Nancy Walker, International Division PCT/US (703) 305-3682	Authorized officer

Form PCT/RO/134 (July 1992)

6. The method of claim 4, wherein the ligand is a CD28Ig/CTLA4Ig fusion protein hybrid.

7. The method of claim 5, wherein the CTLA4Ig fusion protein is CTLA4Ig designated ATCC 68629.

8. The method of claim 1, 2, or 3, wherein the first soluble ligand is a monoclonal antibody reactive with B7 antigen.

9. The method of claim 8, wherein the antibody is anti-BB1 monoclonal antibody.

10. The method of claim 6, wherein the ligand is a CD28Ig/CTLA4Ig fusion protein hybrid having a first amino acid sequence corresponding to a portion of the extracellular domain of CD28 receptor fused to a second amino acid sequence corresponding to a portion of the extracellular domain of CTLA4 receptor and a third amino acid sequence corresponding to the hinge, CH2 and CH3 regions of human immunoglobulin Cg1.

11. The method of claim 1, 2, or 3, wherein the second soluble ligand for the gp39 antigen is a monoclonal antibody reactive with the gp39 antigen.

12. The method of claim 11, wherein the antibody is MR1 monoclonal antibody.

13. The method of claim 4, wherein the extracellular portion of CTLA4 is joined to a non-CTLA4 protein sequence.

14. The method of claim 13, wherein the non-CTLA4 protein sequence is at least a portion of an immunoglobulin molecule.

15. The method of claim 2 or 3, wherein the subject is an animal subject.

16. The method of claim 15, wherein the animal subject is a human.

reaction of the CD28 antigen with endogenous B7, the blockage thereby inhibiting the immune response.

28. The method of claim 27, wherein the soluble ligand of step (a) is a monoclonal antibody directed against CD40.

29. The method of claim 27, wherein the soluble ligand of step (b) is a monoclonal antibody directed against CD28.

30. The method of claim 17, wherein:

a) the step of preventing the endogenous CD40 antigen from binding its endogenous ligand comprises contacting a CD40-positive cell with a soluble ligand which recognizes and binds the CD40 antigen,

b) the step of preventing the endogenous B7 antigen from binding its endogenous ligand comprises contacting a CTLA4-positive cell with a soluble ligand which recognizes and binds the CTLA4 antigen,

the binding of the CD40-positive cell to the soluble ligand of step (a) thereby blocking the reaction of CD40 antigen with endogenous gp39, the binding of the CTLA4- or CD28-positive cell to the soluble ligand of step (b) thereby blocking the reaction of the CTLA4 antigen with endogenous B7, the blockage thereby inhibiting the immune response.

31. The method of claim 30, wherein the soluble ligand of step (a) is sgp39.

32. The method of claim 30, wherein the soluble ligand of step (b) is monoclonal antibody directed against CTLA4.

33. The method of claim 17, wherein:

a) the step of preventing the endogenous CD40 antigen from binding its endogenous ligand comprises contacting a gp39-positive cell with a soluble ligand which recognizes and binds the gp39 antigen,

b) the step of preventing the endogenous B7 antigen from binding its endogenous ligand comprises contacting a CD28-positive cell with a soluble ligand which recognizes and binds the CD28 antigen,

the binding of the gp40-positive cell to the soluble ligand of step (a) thereby blocking the reaction of gp39 antigen with endogenous CD40, the binding of the CD28-positive cell to the soluble ligand of step (b) thereby blocking the

reaction of the CD28 antigen with endogenous B7, the blockage thereby inhibiting the immune response.

34. The method of claim 33, wherein the soluble ligand of step (a) is a monoclonal antibody reactive with the gp39 antigen.

35. The method of claim 33, wherein the soluble ligand of step (b) is monoclonal antibody directed against CD28.

36. The method of claim 17, wherein:

a) the step of preventing the endogenous CD40 antigen from binding its endogenous ligand comprises contacting a gp39-positive cell with a soluble ligand which recognizes and binds the gp39 antigen,

b) the step of preventing the endogenous B7 antigen from binding its endogenous ligand comprises contacting a CTLA4-positive cell with a soluble ligand which recognizes and binds the CTLA4 antigen,

the binding of the CD40-positive cell to the soluble ligand of step (a) thereby blocking the reaction of gp39 antigen with endogenous CD40, the binding of the CTLA4-positive cell to the soluble ligand of step (b) thereby blocking the reaction of the CTLA4 antigen with endogenous B7, the blockage thereby inhibiting the immune response.

37. The method of claim 36, wherein the soluble ligand of step (a) is a monoclonal antibody reactive with the gp39 antigen.

38. The method of claim 36, wherein the soluble ligand of step (b) is monoclonal antibody directed against CTLA4.

39. A pharmaceutical composition useful to inhibit an immune response comprising a pharmaceutically effective amount of a soluble ligand which recognizes and binds a B7 antigen and an acceptable carrier.

40. A pharmaceutical composition useful to inhibit an immune response comprising a pharmaceutically effective amount of a soluble ligand which recognizes and binds a CD28 antigen and an acceptable carrier.

FIG. 1

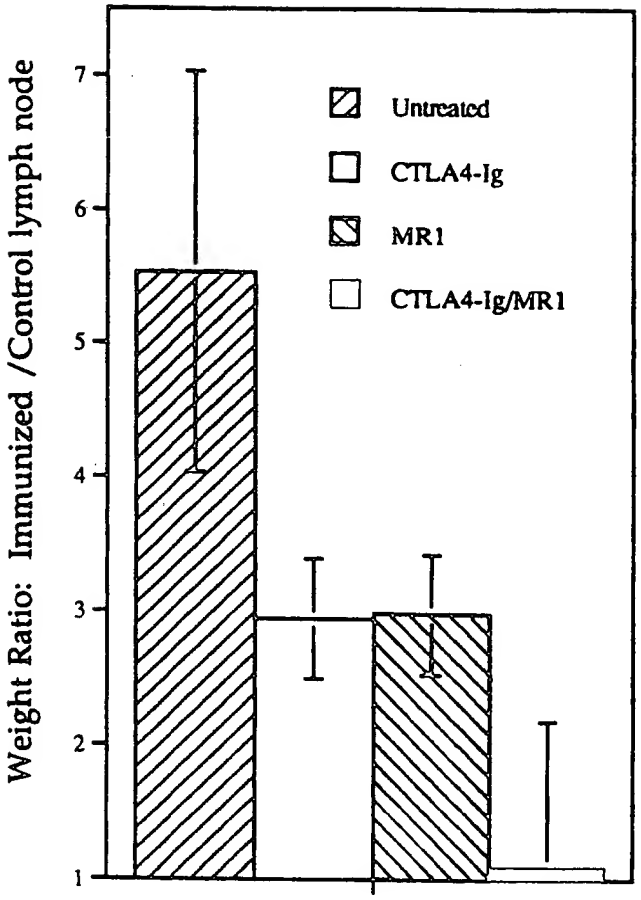

Weight Ratio: Immunized /Control lymph node
7
6
5
4
3
2
1
Untreated
CTLA4-Ig
MR1
CTLA4-Ig/MR1

FIG. 2A

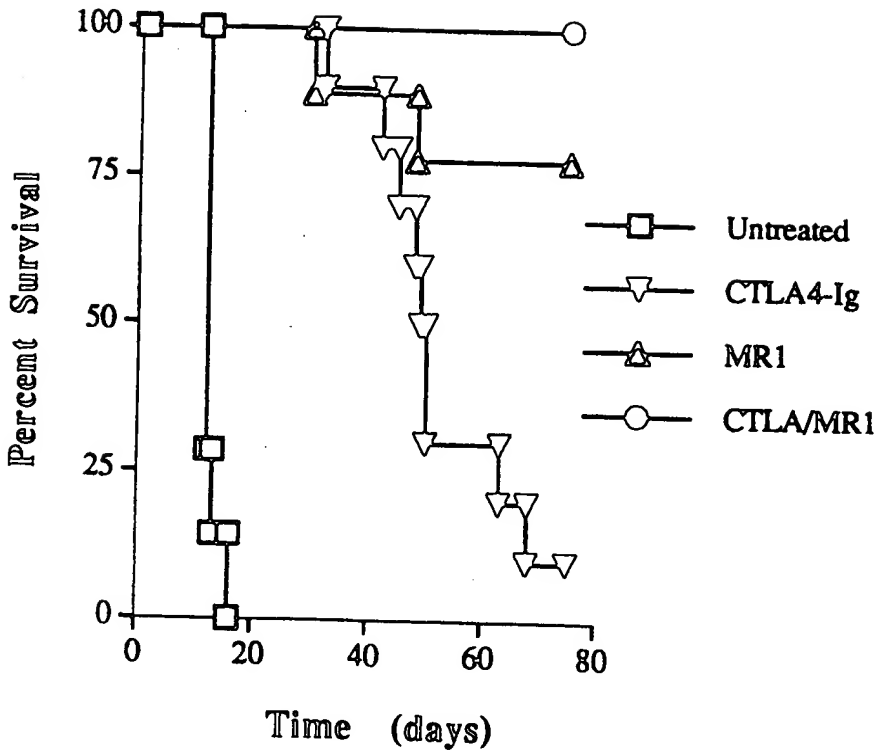

100
75
50
25
0
Percent Survival
0
20
40
60
80
Time (days)
Untreated
CTLA4-Ig
MR1
CTLA/MR1

FIG. 2B

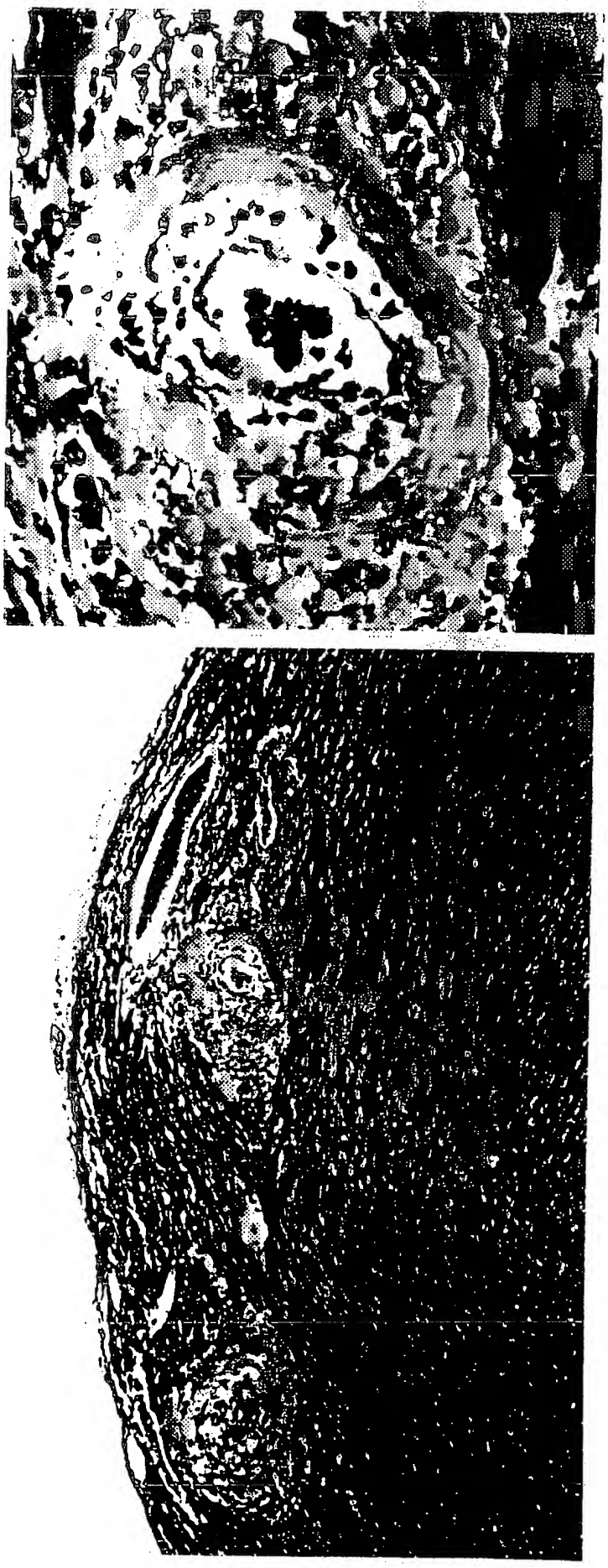


FIG. 2C

FIG. 2D

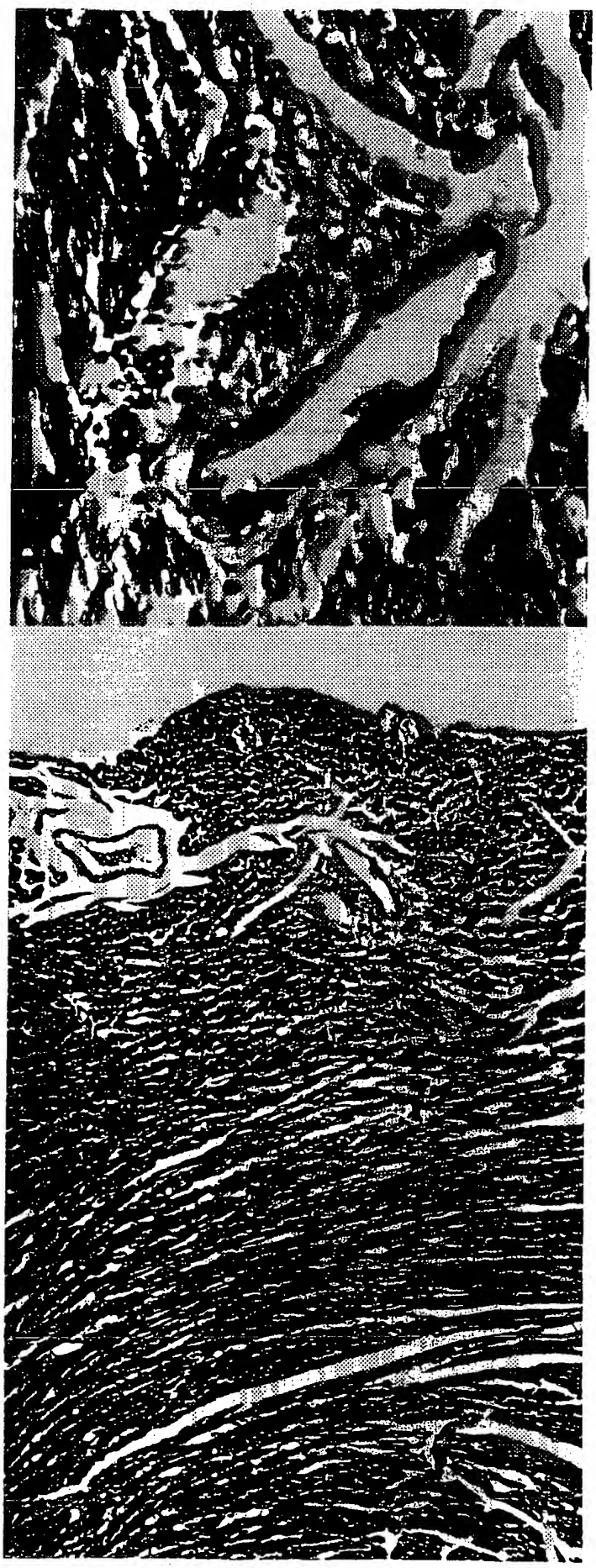


FIG. 2E

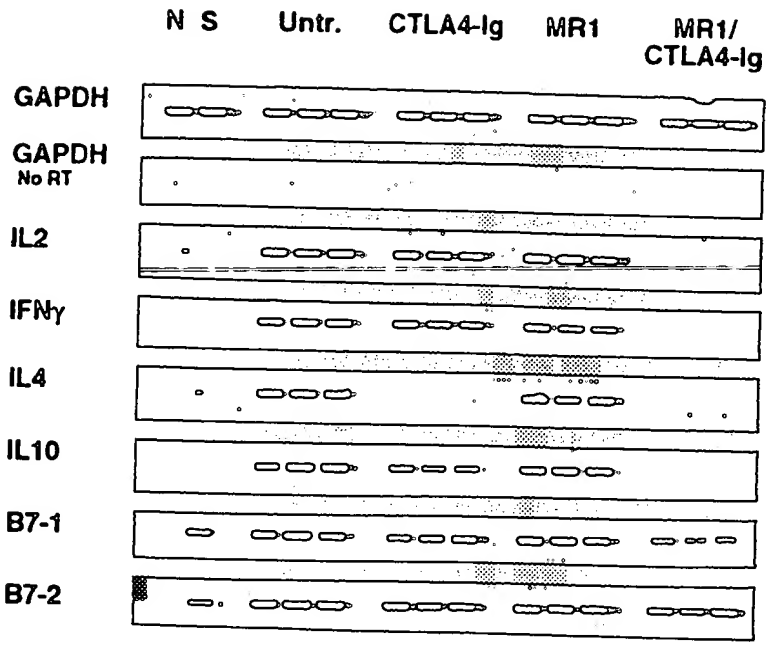

N S
Untr.
CTLA4-Ig
MR1
MR1/
CTLA4-Ig
GAPDH
GAPDH
No RT
IL2
IFNγ
IL4
IL10
B7-1
B7-2

FIG. 3A

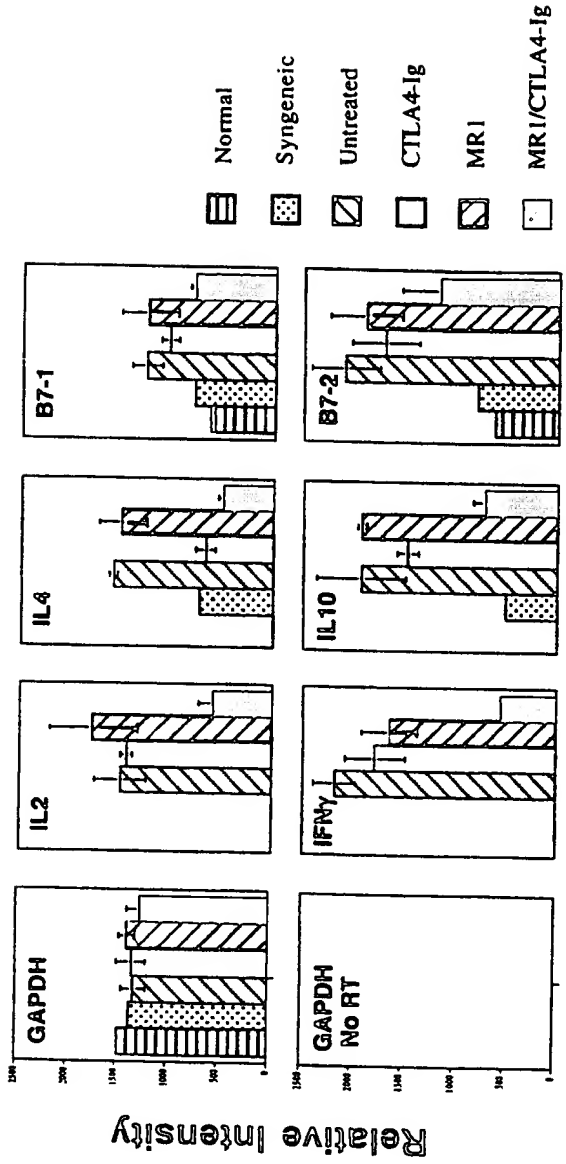
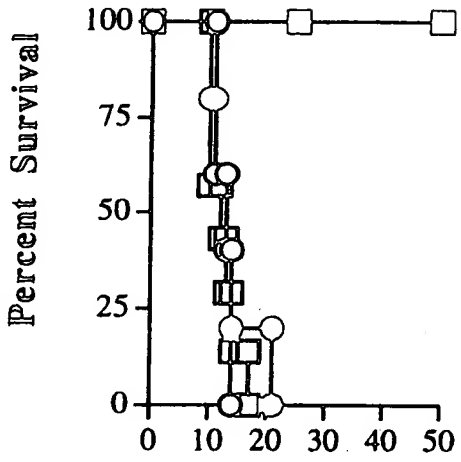

Normal
Syngeneic
Untreated
CTLA4-Ig
MR1
MR1/CTLA4-Ig
B7-1
B7-2
IL4
IL10
IL2
IFNγ
GAPDH
GAPDH No RT
Relative Intensity

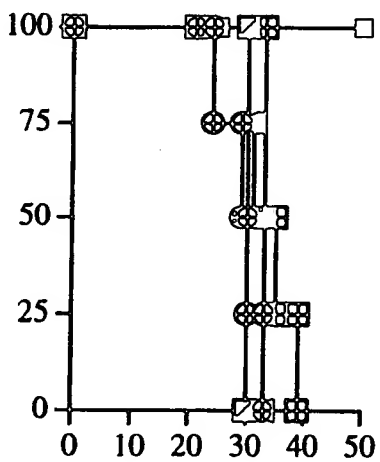
FIG. 3B

FIG. 4A



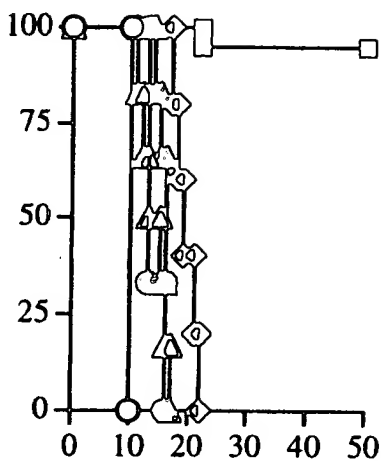
Untreated controls
CTLA4-Ig
MR1
CTLA4-Ig/MR1

FIG. 4B



CyA only
CyA/CTLA4-Ig
CyA/MR1
CyA/CTLA4-Ig/MR1
CTLA4/MR1

FIG. 4C



Untreated controls
MR1
YTS 191
YTS191 + MR1
YTS191 + CTLA4-Ig
CTLA4-Ig + MR1

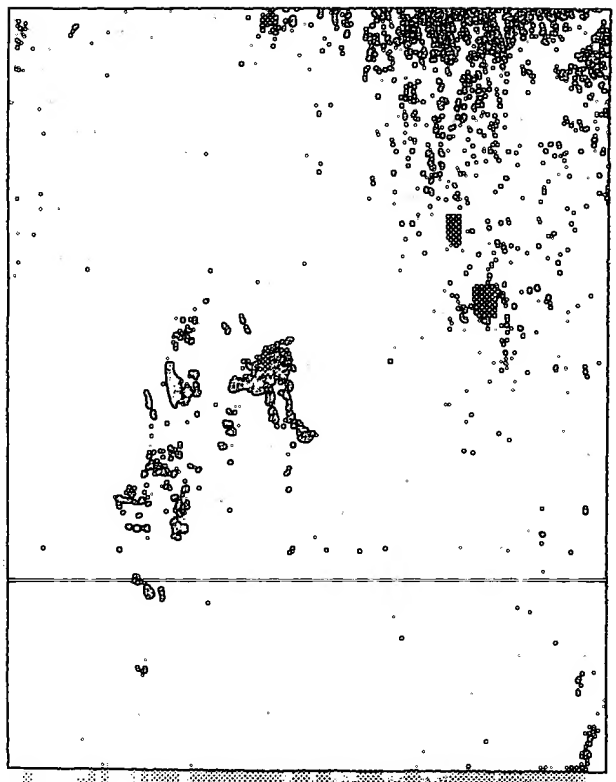


FIG. 4E

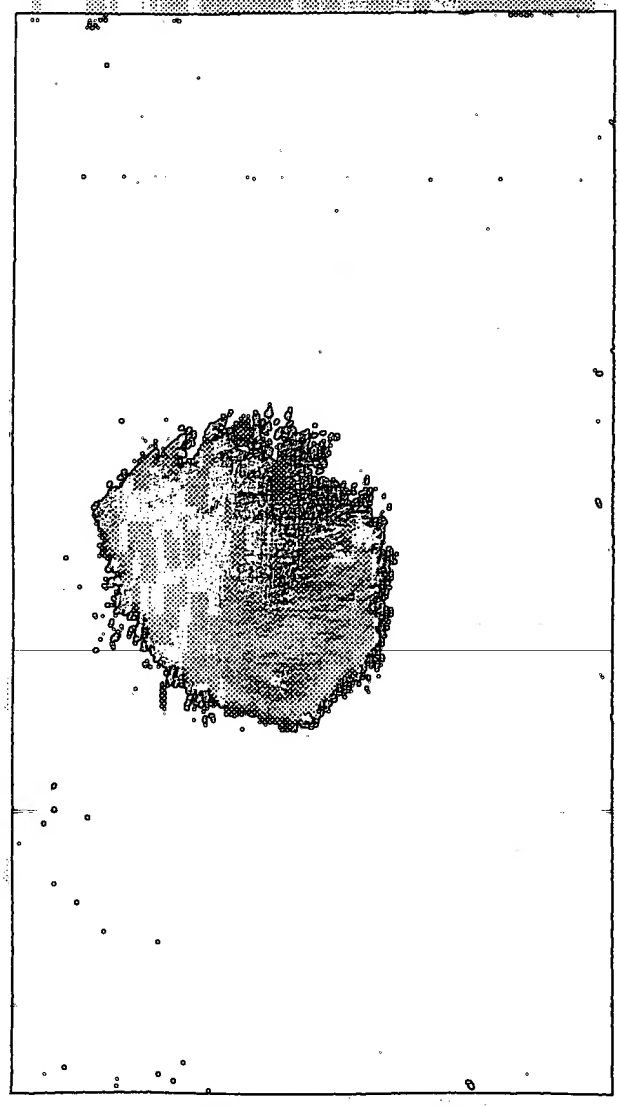


FIG. 4D

FIG. 4G

FIG. 4F

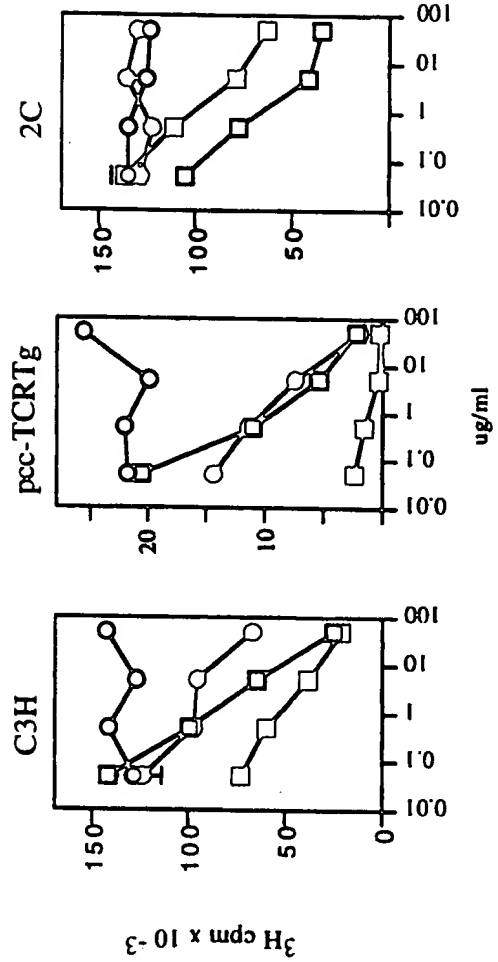

2C
pcc-TCRTg
C3H
150
100
50
20
10
0
100
10
1
0.1
0.01
ug/ml
^{3}H cpm x 10 -3

FIG. 5A

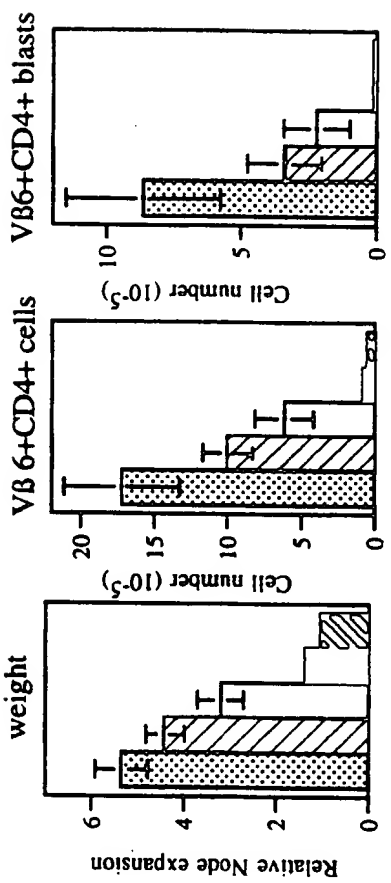

Vβ6+CD4+ blasts
Vβ 6+CD4+ cells
weight
Cell number (10^{-5})
Cell number (10^{-5})
Relative Node expansion
10
5
0
20
15
10
5
0
6
4
2
0

FIG. 5B

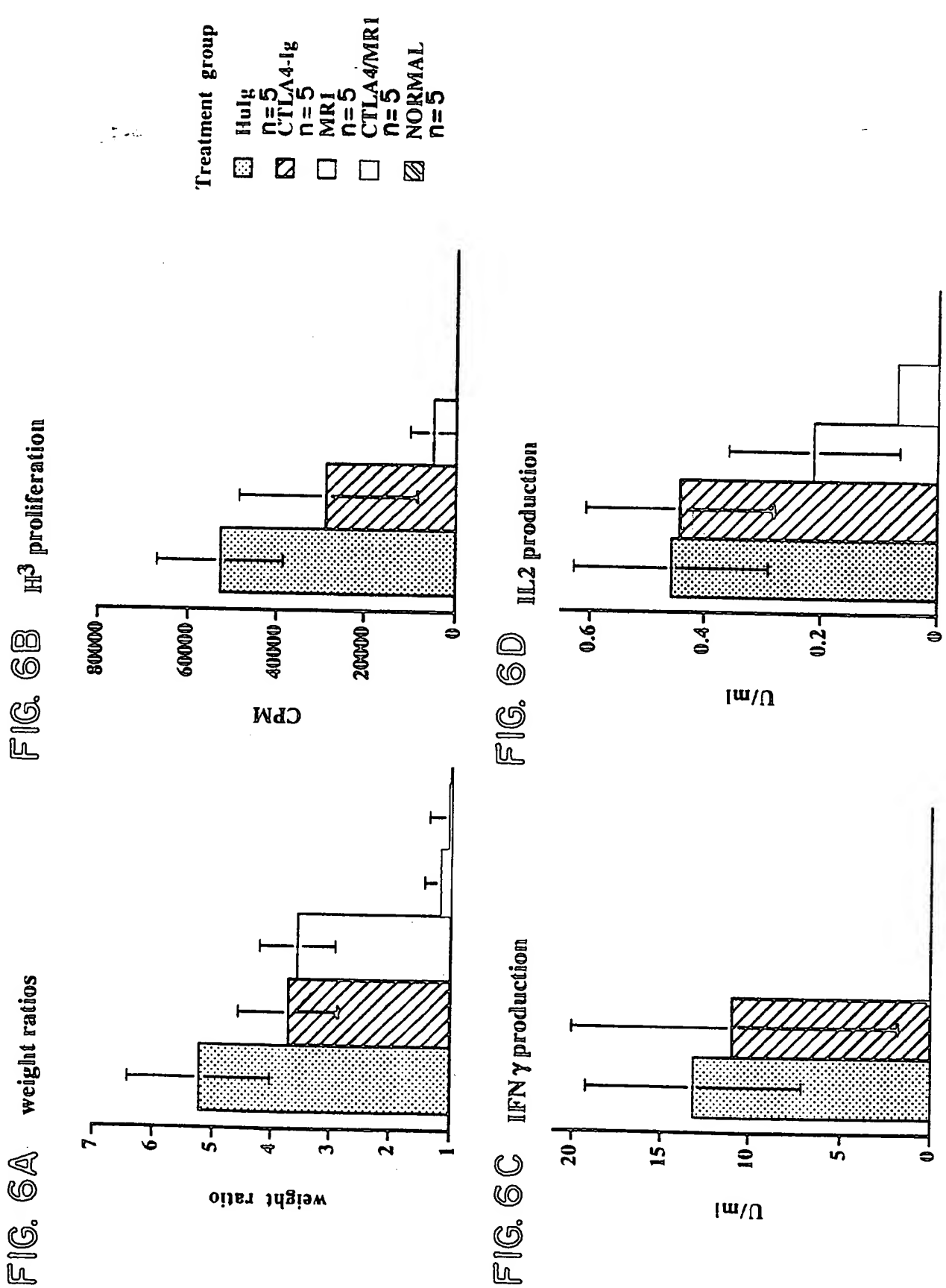


FIG. 6A
weight ratios
weight ratio
7
6
5
4
3
2
1
FIG. 6B
H3 proliferation
CPM
80000
60000
40000
20000
0
Treatment group
Hulg n=5
CTLA4-Ig n=5
MR1 n=5
CTLA4/MR1 n=5
NORMAL n=5
FIG. 6C
IFN γ production
U/ml
20
15
10
5
0
FIG. 6D
IL2 production
U/ml
0.6
0.4
0.2
0

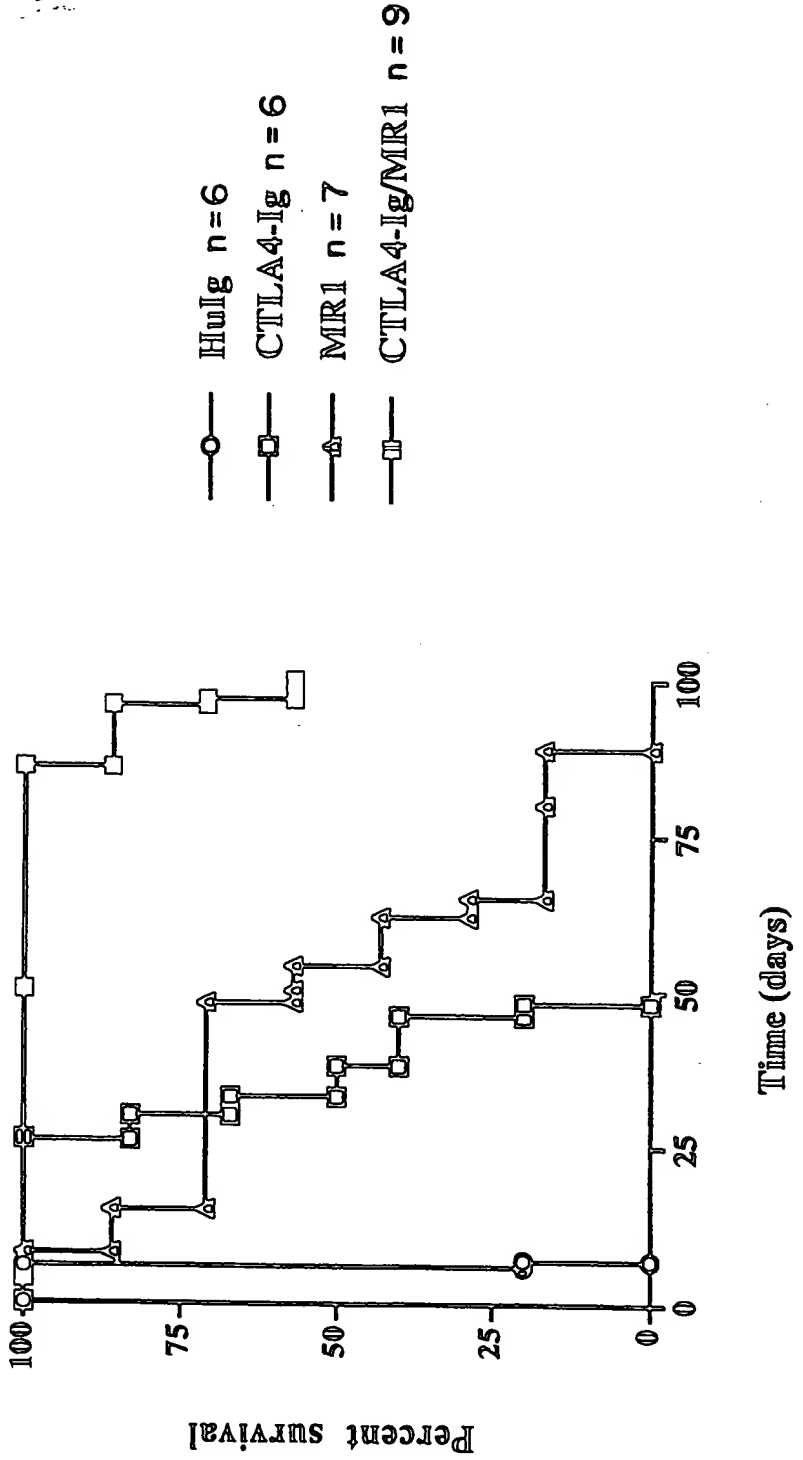

HuIg n=6
CTLA4-Ig n=6
MIR1 n=7
CTLA4-Ig/MIR1 n=9
Percent survival
100
75
50
25
0
0
25
50
75
100
Time (days)

FIG. 7A

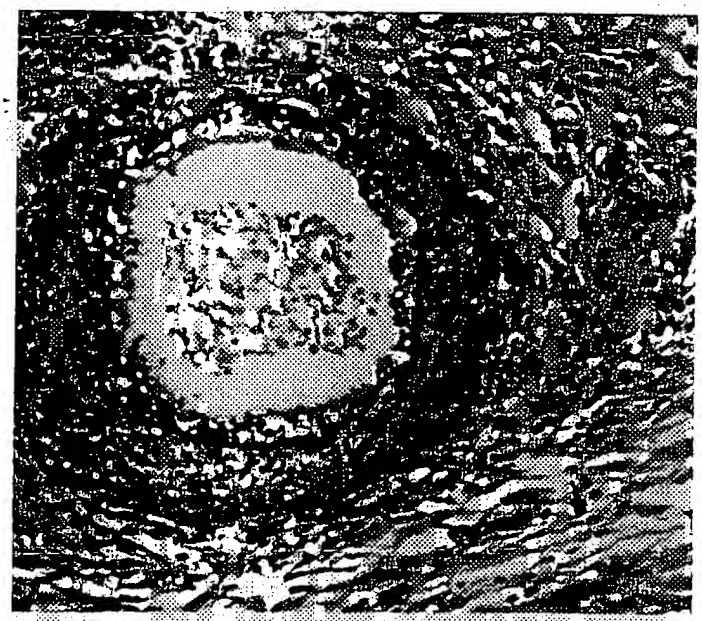


FIG. 7D

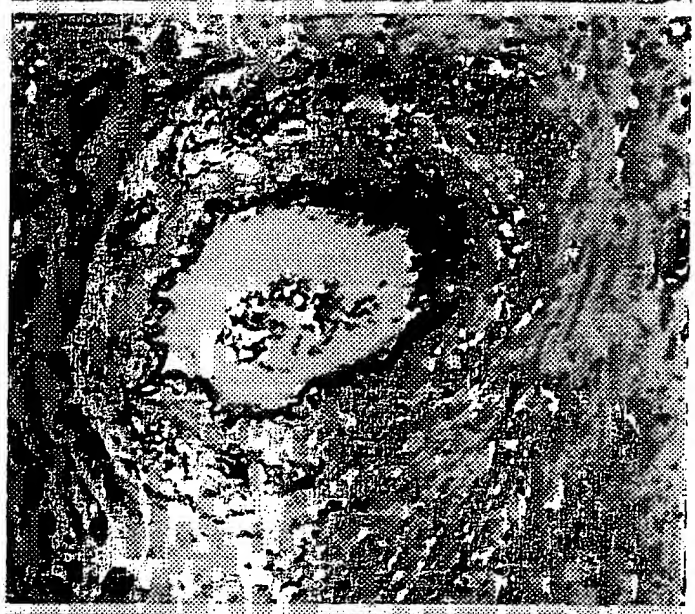


FIG. 7C

FIG. 7B

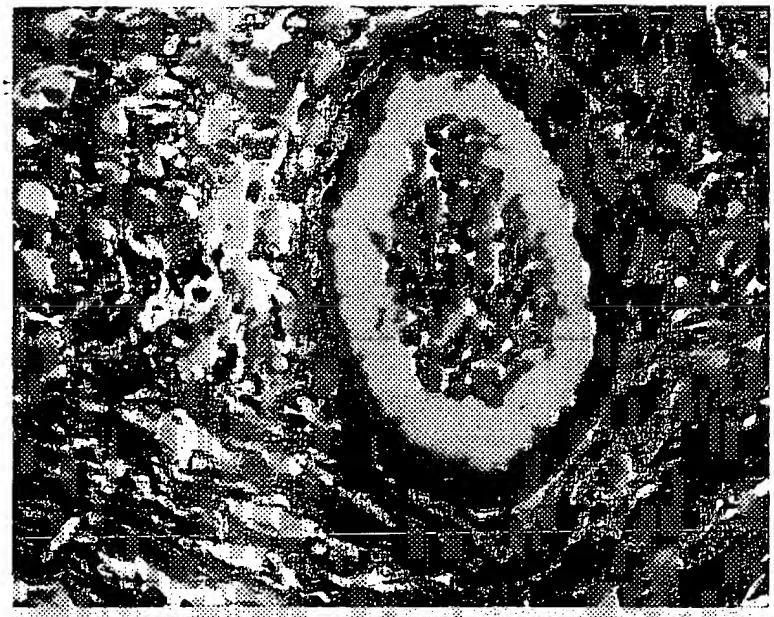


FIG. 7G

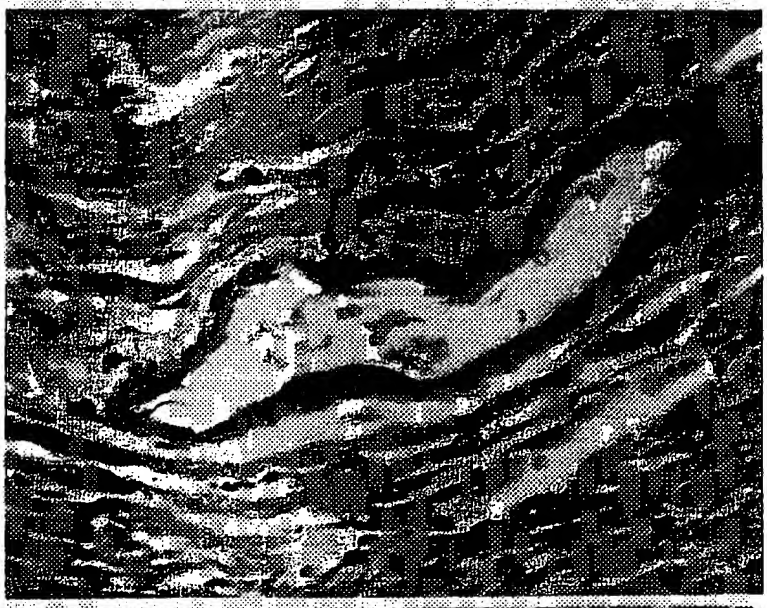
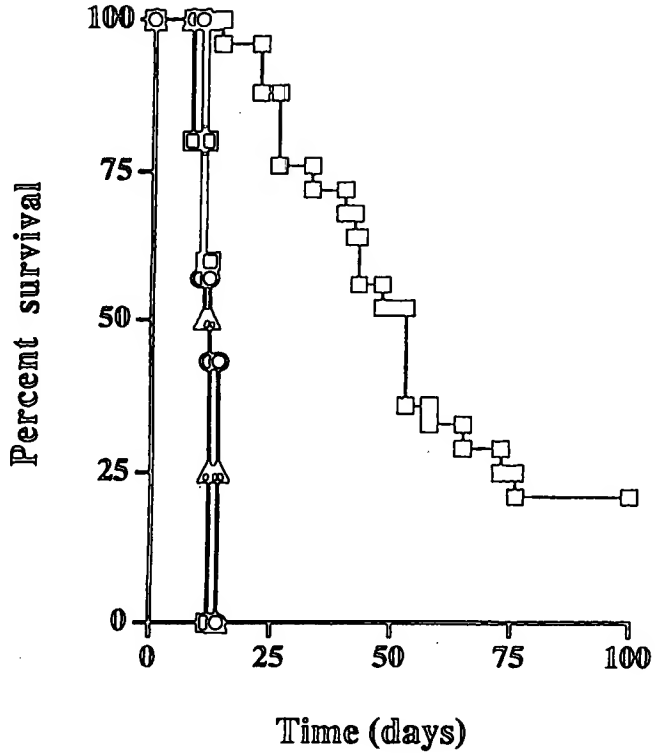


FIG. 7F

FIG. 7E

FIG. 8A



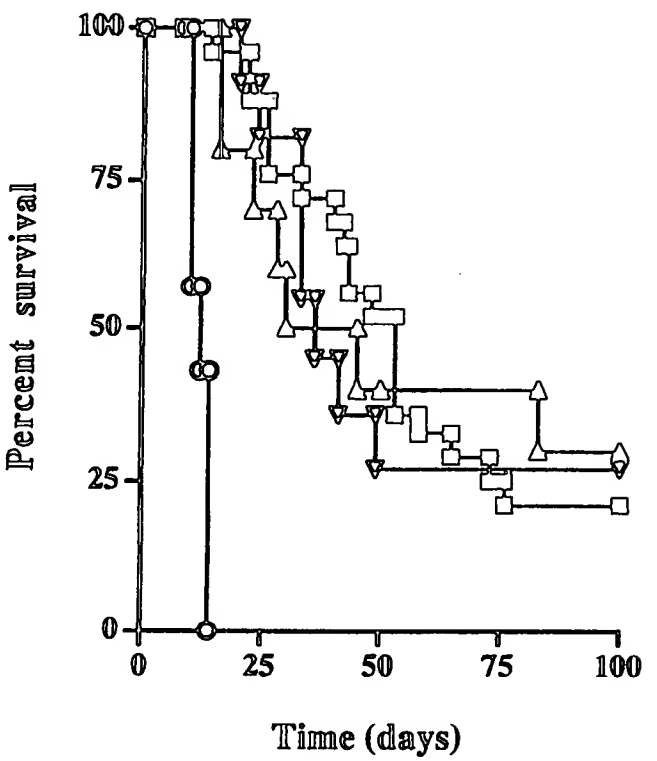
Control n = 8

CTLA4-Ig n = 4

MR1 n = 4

CTLA4-Ig/MR1 n = 25

FIG. 8B



Control n = 8

CTLA4-Ig/MR1 n = 25

chronic CTLA4-Ig/MR1 n = 11

CTLA4-Ig/MR1+chronic MR1 n = 10

FIG. 8D

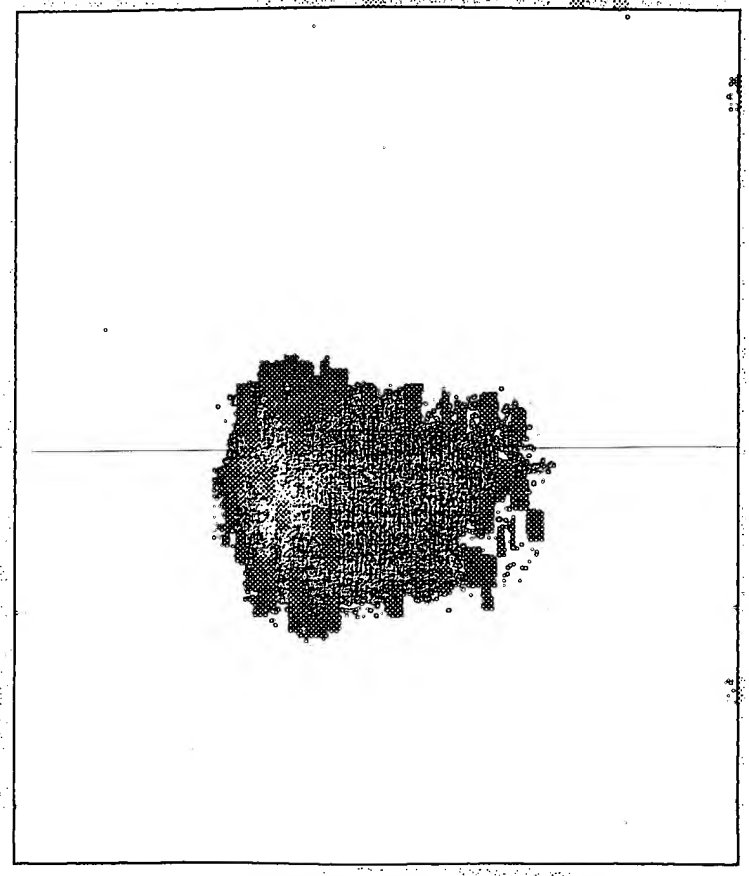


FIG. 8C

FIG. 8F

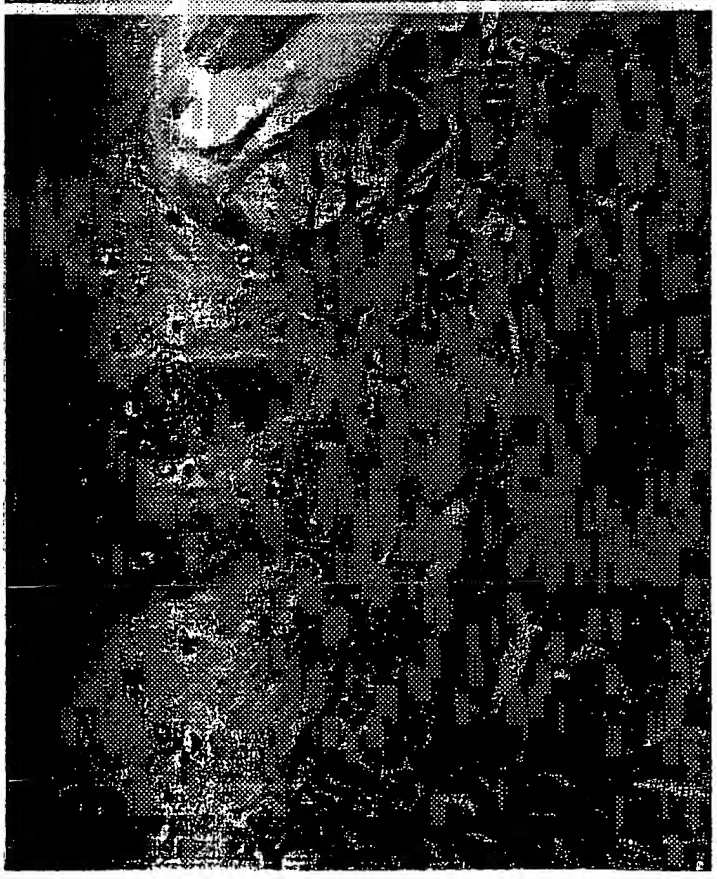


FIG. 8E

Mean Channel Fluorescence
600
500
400
300
200
100
0
Control
CTLA4-Ig
MRI
CTLA4/MRI
Normal
HEART GRAFT RECIPIENTS

FIG. 9B

Mean Channel Fluorescence
600
500
400
300
200
100
0
Control
CTLA4-Ig
MRI
CTLA4/MRI
Normal
SKIN GRAFT RECIPIENT

FIG. 9A

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/395 A61K38/17 //(A61K39/395,38:17)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF CELLULAR BIOCHEMISTRY, SUPPLEMENT, vol. 0, no. 21 part A, 1995, NEW YORK, NY, USA, page 141 XP002037030 N. GRIGGS ET AL.: "Contribution of CD28/CTLA4/B7 and gp39/CD40 costimulation pathways in clonal expansion and functional acquisition of self-reactive T cells." see abstract C2-427 --- -/--	1-50

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 August 1997	26. 08. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THE JOURNAL OF IMMUNOLOGY, vol. 157, no. 1, 1 July 1996, BALTIMORE, MD, USA, pages 117-125, XP002037034 A. TANG ET AL.: "Suppression of murine allergic contact dermatitis by CTLA4Ig. Tolerance induction of TH2 responses require additional blockade of CD40-ligand." see abstract see page 124, left-hand column, line 19 - line 41 see figures 9,10 ---	1-50
P,X	NATURE, vol. 381, no. 6581, 30 May 1996, LONDON, GB, pages 434-438, XP002037035 C. LARSEN ET AL.: "Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways." see abstract -----	1-50

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9200092 A	09-01-92	CA 2086325 A	03-01-92
		EP 0537293 A	21-04-93
		JP 6508501 T	29-09-94
		US 5521288 A	28-05-96
		US 5580756 A	03-12-96
EP 555880 A	18-08-93	AU 3298893 A	19-08-93
		AU 7198096 A	06-02-97
		CA 2089229 A	15-08-93
		JP 6220096 A	09-08-94
		NZ 245898 A	27-04-95
		ZA 9301013 A	20-09-93
WO 9401547 A	20-01-94	US 5397703 A	14-03-95
		CA 2125472 A	20-01-94
		EP 0651797 A	10-05-95
		JP 7509359 T	19-10-95